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Effects of gallic acid on the cell structure of *Xanthomonas oryzae* pv. *oryzicola*

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Abstract: Gallic acid (GA) is a phenolic compound, and presents various biological activities in plants. Our previous experiments demonstrated a relatively strong inhibitory effect of GA on *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*). In order to elucidate the effects of GA on the cell structure and membrane permeability of the pathogen, morphologies of bacteria treated with GA were observed by electron microscopy, the integrity and the permeabilities of membrane of *Xoc* were investigated by determining the release from cells of materials that absorb at 260 nm, changes in the fluorescence of cells treated with the fluorescein diacetate(FDA) and lactate dehydrogenase(LDH) activity. Treatment with 200 $\mu g \cdot mL^{-1}$, many pits and irregular vesicles were observed on the cell surface under electron microscopy, indicating that GA could damage the cell walls of *Xoc*. The electrical conductivity from *Xoc* suspensions 24 h after GA treatment was 135.48 $\mu S \cdot cm^{-1}$（Control was 127.85 $\mu S \cdot cm^{-1}$）, and the fluorescence intensity of *Xoc* suspensions 2 h after GA decreased by 58.10%, indicating that cells leaked electrolytes and cytosolic contents. Meanwhile, the activities of LDH in bacterial suspensions treated with GA also increased, suggesting that GA could damage the structure of the bacterial cell membrane. In addition, the absorbance at 260 nm from *Xoc* suspensions was 1.004（control was 0.018）, indicating that GA could negatively affect *Xoc* cell wall integrity. These results indicate that GA not only altered the permeability of the cell membrane of *Xoc*, but also impacted the integrity of the cell membrane.

Key words: *Xanthomonas oryzae* pv. *oryzicola*, gallic acid, cell structure, membrane permeability, bacteriostasis mechanism

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没食子酸对水稻细菌性条斑病菌细胞结构的影响

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摘要：没食子酸（gallic acid，GA）是一种植物酚类化合物，具有多种生物活性。本研究的前期试验发现，GA 对水稻细菌性条斑病菌（*Xanthomonas oryzae* pv. *oryzicola*，*Xoc*）具有较强的抑制作用。为了解该物质对 *Xoc* 的细胞结构和细胞膜的影响，本研究用电子显微镜观察 GA 对 *Xoc* 的形态结构的影响，通过测定 GA 处理后的 *Xoc* 培养液的电导率、紫外吸收物含量（260 nm 的吸光值）、乳酸脱氢酶的活性以及菌体的二乙酸荧光素（fluorescein diacetate，FDA）的强度等探讨 GA 对 *Xoc* 细胞膜的完整性和通透性的影响。试验结果显示，经浓度为 200 μg·mL^{-1} 的 GA 处理后，*Xoc* 的菌体形态结构发生改变，表面有明显的凹陷或不规则囊泡状突起，表明 GA 对 *Xoc* 细胞壁有损伤作用。200 μg mL^{-1} 的 GA 处理 24 h 后，病菌培养液的电导率为 135.48 μS cm^{-1}（对照处理为 127.85 μS·cm^{-1}），GA 处理 2 h 后，*Xoc* 细胞荧光强度下降 58.10%，说明病菌细胞内电解质外渗和细胞溶质发生渗漏；同时，乳酸脱氢酶的活性增加，表明菌体的细胞膜受到破坏。此外，GA 处理 24 h 后，*Xoc* 培养液在 260 nm 下的吸光值为 1.004（对照处理为 0.018），表明病菌细胞膜的完整性受到破坏。研究结果表明，GA 不仅破坏 *Xoc* 的细胞膜通透性，而且也影响膜的完整性。

关键词：水稻细菌性条斑病菌，没食子酸，细胞结构，膜通透性，抑菌机制

Bacterial leaf streak of rice, which is caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), is one of the important plant diseases in South China. This disease was first reported in Philippines in 1918 and was reported in China in 1953. Bacterial leaf streak of rice can reduce yield loss by 40%-60%, and the disease severely threatens the high and stable yield production of rice. Furthermore, the transportation of rice seeds during production tends to expand and aggravate this disease (Zhang et al, 2015). Because bacterial leaf streak-resistant rice cultivars are not available, bactericides are mainly used to control the disease. However, few bactericides, such as zinc thiazole and thiodiazole copper, are registered in China (Wei et al, 2007). Therefore, the development of new bactericides to manage this disease is desirable.

Gallic acid [GA: 3,4,5-trihydroxybenzoic acid ($C_6H_2(OH)_3COOH$] is a phenolic compound in plants that exists as a free molecule and as a constituent of tannins (Masoud et al, 2012). Studies have demonstrated the antimicrobial activity of GA against *Salmonella typhimurium* (Nohynek et

al, 2006), *Escherichia coli* (Chanwitheesuk et al, 2007), *Bacillus subtilis* (Zhang et al, 2013), *Staphylococcus aureus* and *Candida albicans* (Sarjit et al, 2015). GA may effectively inhibit the invasion ability of gastric cancer MGC-803 cells (Li et al, 2017).Our early work involving the screening of plants for antibacterial activity against *Xoc* showed that GA extracted from *Sedum lineare* significantly inhibited the growth of plant pathogens such as *Xoc*, *X. oryzae* pv. *oryzae*, *X.campestris* pv. *pruni*, *X. axonopodis* pv. *citri*, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *glycinea*, *P. syringae* pv. *tomato* and *Pectobacterium carotovora* subsp. *carotovora in vitro*.

Reports on the antibacterial mechanisms of GA exist. GA damages the cell membrane of *Pseudomonas fluorescens*, which induced the leakage of intracellular electrolytes and abundant molecular material (Lu et al, 2015). GA may potentially capture calcium ions from the calcium-binding proteins on the cell surfaces of *Campylobacter* by chelation, resulting in the loss of vital functions of those proteins and therefore cell death (Sarjit et al, 2015). GA inhibits the proliferation of human hepatocellular carcinoma SMMC-7721 cells by inhibiting the expression of Survivin mRNA (Li et al, 2014), inhibits the expression of MMP2 and MMP9 by regulating PI3K / AKT signaling pathway, and also effectively inhibits plankton SH FurnRi activity and biofilm formation by regulating the expression of MDOH gene and OPGH protein (Kang et al, 2018). GA plays an antibacterial role by destroying the membrane integrity of *Aeromonas hydrophila* and aeromonas cold blood (Lu et al, 2016).To our knowledge, no reports on the mechanisms of GA against *Xoc* have been published. In the present paper, we evaluated the minimum inhibitory concentration (MIC) of GA against *Xoc* and the effects of GA on the cell structure and membrane permeability of the pathogen. The results will provide a theoretical basis for further GA applications.

1 Materials and methods

1.1 Materials

GA was obtained from the Tianjin Kemiou chemical reagent development center. An assay kit for determining the activity of lactate dehydrogenase (LDH) was obtained from Suzhou Comin Biotechnology Co, Ltd, China. Methanol, ethyl acetate, glutaraldehyde, iodoacetic acid, sodium phosphate, propanedioic acid, phenol, fluorescein diacetate (FDA), 2,4-dinitrophenylhydrazine, trichloroacetic acid, sodium carboxymethyl cellulose, sodium polypectinate, sodium hydroxide and absolute alcohol were of the highest grade commercially available.

1.2 Bacterial strains and growth conditions

Xoc strain Xo-002 was isolated from an infected rice leaf in 2010 in Guangxi and was preserved at the Plant Pathology Research Institute of Guangxi University, Nanning, China. The strain was stored on nutrient agar (NA: 3 g of beef extract, 5 g of peptone, 10 g of dextrose, 17 g of agar, 1 000 mL of distilled water, pH 7.0) at -80 ℃ and was initially cultured on NA at 30 ℃

for 2 days, after which the strain was transferred into beef extract broth and shaken at 120 r min^{-1} at 28 ℃ for 24 h (during the logarithmic phase).

1.3 Antimicrobial susceptibility tests

Minimum inhibitory concentration (MIC) was tested using a 2-fold serial broth dilution as described here. *Xoc* at the logarithmic growth phase were diluted in a 10^7 colony-forming units (CFU) mL^{-1} suspension of beef extract. GA dissolved in 10% methanol was added to the bacterial suspension to a final GA concentration ranging from 3.125 to 400 μg·mL^{-1}. The bacterial suspension with only 10% methanol served as the control. The suspension was then shaken at 120 r min^{-1} at 30 ℃ for 24 h. The MIC was defined as the lowest concentration of antimicrobial agent at which the cell growth was not visible with the naked eye. Each treatment was replicated three times.

To determine the effects of initial bacterial density on the GA inhibition, *Xoc* at the logarithmic growth phase were diluted to 10^6 CFU mL^{-1}, 10^7 CFU mL^{-1} or 10^8 CFU mL^{-1} in suspension of beef extract. GA was added to the bacterial suspension until final GA concentrations of MIC or 2 MIC were reached. The suspension was then shaken at 120 r min^{-1} 30 ℃ for 24 h. Each treatment was replicated for three times.

1.4 Electron microscopy

Xoc at the logarithmic growth phase were diluted with beef extract to 10^8 CFU mL^{-1}. GA (dissolved in 10% methanol) was added to the bacterial suspension to reach a final GA concentration of MIC. The bacterial suspension with only 10% methanol served as the control. After shaking at 120 r min^{-1} and 28 ℃ for 6, 12 or 24 h, the suspension was centrifuged. The cells were washed three times with 0.2 M sodium phosphate buffer (PBS, pH 7.4) and then fixed with 2.5% glutaraldehyde in 0.2 M PBS. The samples were prepared for electron microscopy as previously described (Nakajima et al, 2003). The prepared samples were examined using an electron microscope (H-500, Hitachi, Japan)

1.5 Electrical conductivity assay

Pathogens were diluted with beef extract to 10^8 CFU mL^{-1}. GA was added to the bacterial suspension to reach final GA concentrations of 1/2 MIC, MIC or 2 MIC. The bacterial suspension with only 10% methanol served as the control. After shaking at 120 r min^{-1} at 28 ℃ for 0, 1, 2, 4, 8 or 24 h, the suspension was centrifuged, after which the supernatant was diluted 20 times with water and measured for its electrical conductivity. Each treatment was replicated three times.

1.6 Cell membrane integrity assay

The cell membrane integrity of *Xoc* was evaluated by determining the release of materials that absorb at 260 nm. Bacteria at the logarithmic growth phase were washed three times with PBS and then diluted with PBS to 10^8 CFU mL^{-1}. GA was added to the bacterial suspension to

reach final GA concentrations of 1/2 MIC, MIC or 2 MIC. The suspension with only 10% methanol served as the control. Each treatment was replicated three times. The suspension was shaken at 130 r·min^{-1} at 28 ℃ for 0, 2, 4, 8, 16 or 24 h, after which it was centrifuged at 4 000 g for 10 min. The release of materials that absorb at 260 nm was monitored over time using a UV-Visible spectrometer (Shimadzu UV-1800; Shimadzu Corp, Kyoto, Japan)

1.7 Outer membrane (OM) permeabilization assay

The OM permeabilization activity of cell was determined using an FDA assay as described by Zeng et al(2013). *Xoc* at the logarithmic growth phase were diluted with beef extract to a 10^8 CFU·mL^{-1}. GA was added to the bacterial suspension to reach a final GA concentration of MIC. The suspension with only 10% methanol served as the control. After shaking at 130 r·min^{-1} at 28 ℃ for 2 h, the suspension was centrifuged. The cells were washed three times with PBS. FDA was then added to the bacterial suspension to reach a final FDA concentration of 0.25 μg·mL^{-1}, and the suspension was incubated at room temperature for 10 min. Fluorescence excited at a wavelength of 460 nm and an emission wavelength of 680 nm was recorded using an RF-5301PC fluorescence spectrophotometer (Shimadzu, Japan). All experiments were replicated three times.

1.8 Determination of the activity of LDH in the bacterial suspension

Xoc at the logarithmic growth phase were diluted with beef extract to 10^8 CFU·mL^{-1}. GA was added to the bacterial suspension to reach a final GA concentration of 100, 200 or 400 μg·mL^{-1}. The bacterial suspension with only 10% methanol served as the control. After being exposed to GA for 0, 2, 4, 8, 16 or 24 h, 2 L of culture was centrifuged at 1 000 × g for 5 min. The LDH activity in the supernatant was determined using an assay kit (Suzhou Comin Biotechnology Co., Ltd, China) according to the manufacturer's instructions.

All experiments were replicated three times. The formation of 1 nmol of pyruvic acid upon the exposure of 1 mg of bacterial protein to extracellular matrix for 15 min was defined as 1 unit of activity in the reaction systems.

Protein concentration was determined in accordance with the coomassie brilliant blue staining method. A 3 mL coomassie brilliant blue color reagent was added to 0.05 mL of distilled water, 0.05 mL of standard protein solution and 0.05 mL of sample liquid, which represented a control tube, standard tube and testing tube, respectively, for 10 min. The tubes were then and measured for their optical density at 595 nm (OD_{595}) nm using a UV spectrophotometer, and the protein content was calculated as follows:

$$\text{Protein content}(\text{mg} \cdot \text{mL}^{-1}) = \frac{\text{Standard bube concentration} \times (\text{test tube OD} - \text{Standard tube})}{(\text{Standard bube OD} - \text{Standard tube})}$$

1.9 Statistical analysis

The data were subjected to the analysis of variance using SAS software (version 6.08, SAS Institute, Cary, NC). Mean comparisons were conducted using a least significant difference (Fisher's LSD) test at $P = 0.05$. The standard error and LSD results were recorded.

2 Results

2.1 MIC of GA on *Xoc*

Xoc cultured for 24 h grew in NA containing 3.125 μg·mL^{-1} to 100 μg·mL^{-1} GA (Table 1). However, the culture solution was transparent in the NA supplemented with 200 μg·mL^{-1} GA, which indicates that the MIC of GA was 200 μg·mL^{-1}.

Table 1 MIC of GA on *Xoc*

GA concentration (μg·mL^{-1})	Bacterial concentration (CFU mL^{-1})		
	10^6	10^7	10^8
200	-	-	++
400	-	-	+
0	+++	+++	+++

Note: "+++" represents bacterial concentration > 10^{10} CFU mL^{-1}, "++" represents bacterial concentration ranging from 10^8-10^{10} CFU mL^{-1}, "+" represents bacterial concentration < 10^8 CFU mL^{-1}, "-" represents sterile growth. The same below.

The effects of initial bacterial density on GA inhibition were shown in Table 2. Initial bacterial densities of 10^6 CFU mL^{-1} and 10^7 CFU mL^{-1} inhibited the growth of *Xoc* under conditions of 200 μg·mL^{-1} and 400 μg·mL^{-1} GA. However, an initial bacterial density of 10^8 CFU mL^{-1} resulted in turbid culture media when treated with 200 μg·mL^{-1} and 400 μg·mL^{-1} GA, indicating *Xoc* growth.

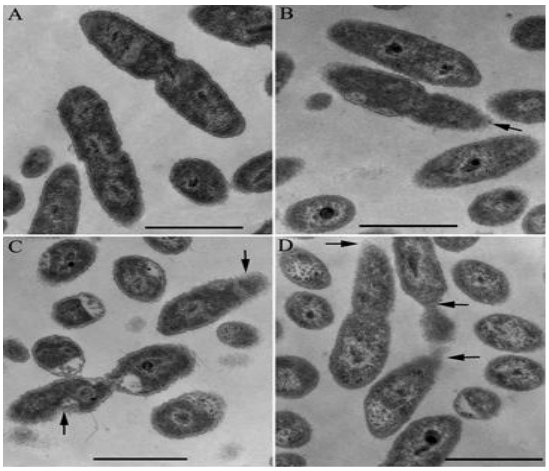
Table 2 Effects of initial bacterial density on GA inhibition

GA concentration (μg·mL^{-1})	Bacterial concentration (CFU mL^{-1})		
	10^6	10^7	10^8
200	-	-	++
400	-	-	+
0	+++	+++	+++

2.2 Effects of GA on *Xoc* cell morphology

The effect of GA on the cellular structure of the *Xoc* was visualized using transmission electron microscopy (Fig.1). Control cells cultured for 12 h had a full shape and

smooth surface; the cell membrane adhered to the cell wall, and the cytoplasm was homogeneous (Fig.1:A). However, GA-treated *Xoc* showed deformation and roughness of cell walls (Fig.1:B,C) after 6 h and 12 h, the cell outlines appeared blurry, the cell wall was loose after 24 h, and some cells had an irregular shape and no membrane or cell wall on one side (Fig.1:D).



Note：**A**. 12 h control with only 10% methanol (CK); **B**. 6 h after GA treatment; **C**.12 h after GA treatment; **D**. 24 h after GA treatment (Bars = 1 μm). The same below.

Fig. 1 Transmission electron microscopy images of normal *Xanthomonas oryzae* pv. *oryzicola* and those affected by GA

Scanning electron microscopy (SEM) showed that *Xoc* cells treated with GA at the MIC changed remarkably compared with control cells (Fig.2). The surface of control cells was smooth and cells were obvious after 12 h of culture (Fig.2:A). However, granules and holes were observed on the surface of GA-treated cells after 6 h and 12 h (Fig.2:B,C), and many pits and irregular vesicles were observed on the cell surface after 24 h (Fig.2:D). Therefore, GA could damage the cell wall of *Xoc*, and the effect was greater with the duration of treatment.

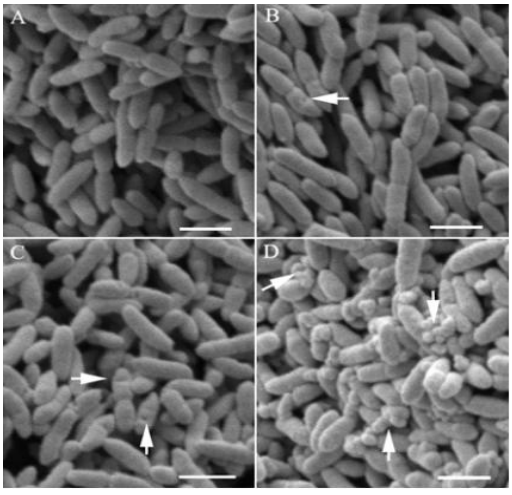
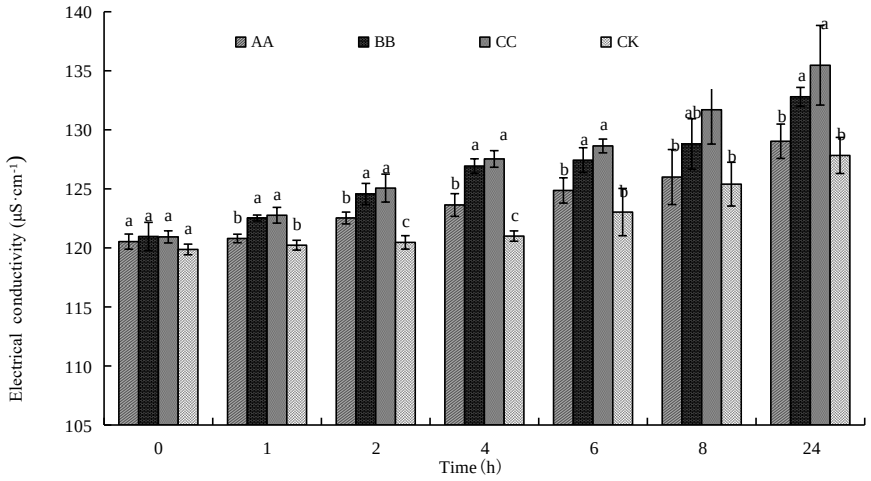


Fig. 2 SEM images of normal *Xanthomonasoryzae* pv. *oryzicola* and those affected by GA

2.3 Effects of GA on the electrical conductivity of bacteria culture media

The effect of GA on the electrical conductivity of the culture solution is shown in Fig.3. Electrical conductivity increased with time. GA treatments at concentrations of 100 μg·mL^{-1}, 200 μg·mL^{-1}, and 400 μg·mL^{-1} and the control culture liquid resulted in electrical conductivities of 129.03, 132.80, 135.48 and 127.85 μS·cm^{-1}, respectively, after 24 h of incubation. The result indicated that the higher the concentration of GA was, the more serious the leakage of electrolytes from the cell.



Note: **AA**.100 μg·mL^{-1}; **BB**. 200 μg·mL^{-1}; **CC**.400 μg·mL^{-1}; **CK** .Only 10% methanol. Columns with different lowercase letters represent significant differences at 0.05. The same below.

Fig. 3 Effects of GA on the electrical conductivity of the culture liquid of *Xoc*

2.4 Effects of GA on the content of UV-absorbing compounds of bacterial culture media

When antimicrobial agents damage bacterial membranes, intracellular components, such as DNA and RNA, tend to leak. The release of DNA and RNA from cells can be detected by UV at 260 nm as an indication of membrane damage (Chen & Cooper, 2002). The content of UV-absorbing compounds from *Xoc* suspensions treated with GA is shown in Fig. 4. GA treatment concentrations altered the absorbance: the higher the GA treatment concentration was, the larger the absorbance. Treatment with 100, 200 and 400 μg·mL^{-1} GA for 24 h resulted in absorbances of 0.195, 1.004 and 1.720, respectively, and the difference between GA treatment and the control (0.018) was statistically significant. This result indicated that GA could negatively affect *Xoc* cell wall integrity.

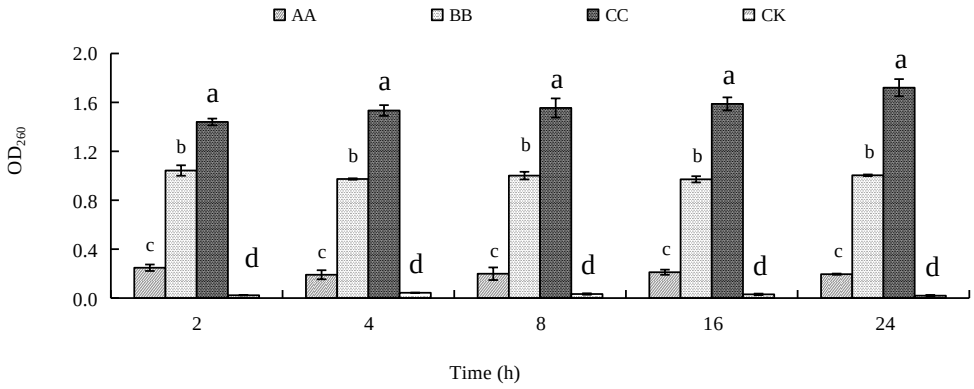


Fig. 4 Effects of GA on the efflux of UV-absorbing materials in the culture liquid of *Xoc*

2.5 Effects of GA on the permeability of bacteria

FDA, which can enter the cell, is a nonfluorescent compound that is hydrolyzed to fluorescein and acetate by nonspecific esterases. The intracellular retention of fluorescein depends on the integrity of the cell membrane (Prud êncio et al, 1998). The change in fluorescence intensity after GA (200 μg·mL^{-1}) treatment for 2 h was presented in Fig. 5. Stained cells displayed the most intense fluorescence at 510 nm. The fluorescence intensities of both 200 μg·mL^{-1} GA-treated and control cells were 53.06% and 91.33%, respectively. In addition, the fluorescence intensity of the treated cells decreased by 58.10%, and the cytosolic contents had leaked out from the cells.

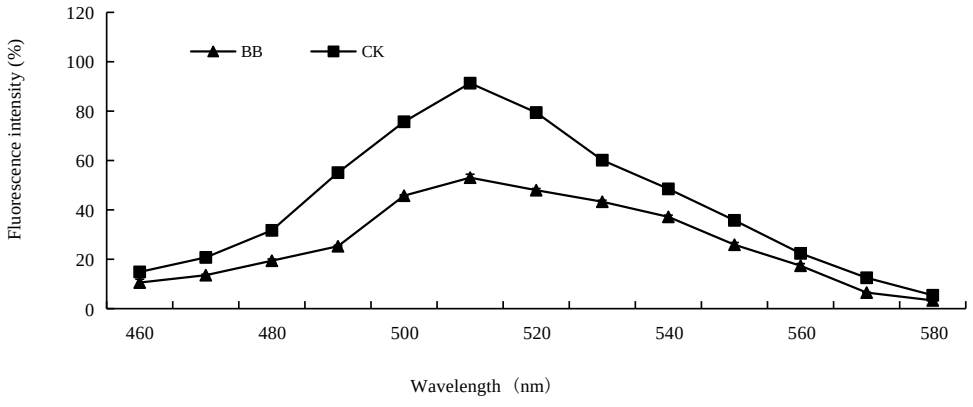


Fig. 5 Effects of GA on the fluorescence intensity of *Xoc* using FDA staining

2.6 Effects of GA on the activity of the LDH of bacteria

The bacterial endoenzyme LDH could leak from bacteria upon cell membrane damage. Therefore, the activity of LDH in the bacterial suspension reflects the change in permeability of the cell membrane (Hara & Yamakawa, 1995). The activities of LDH in the bacterial suspensions were shown in Fig. 6. The activities of LDH in bacterial suspensions treated with GA (100, 200 and 400 μg mL^{-1}) and the control were less than 1.5 U mg^{-1} protein after treatment for 2 h; there was no significant difference between the GA treatment and the control. However, the enzymatic activities of GA-treated suspensions were higher than those of the control after 4 h. The enzymatic activities increased with GA dose and duration. The results suggested that GA could damage the structure of the bacterial cell membrane and caused the leakage of LDH enzymes.

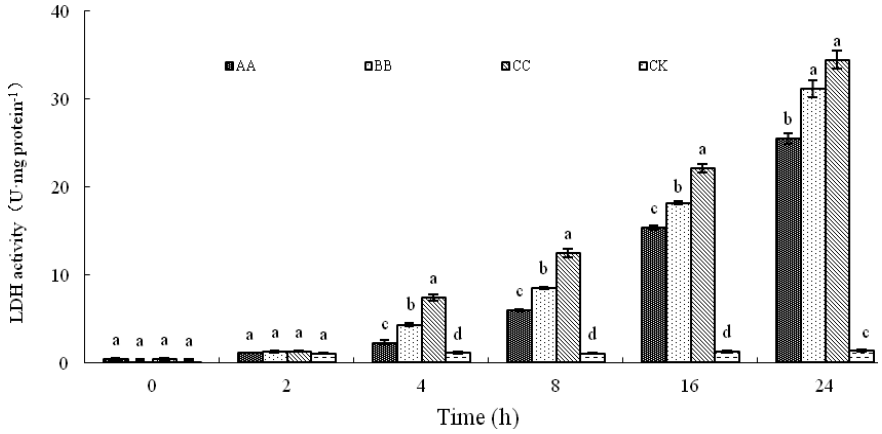


Fig. 6 Effects of GA on LDH activity in the culture liquid of *Xoc*

3 Discussion

A concentration of 200 μg mL^{-1} GA completely inhibited cells at 10^7 CFU mL^{-1}; therefore, the MIC of GA on *Xoc* was 200 μg mL^{-1} in these conditions. The antibacterial function of GA differed against different concentrations of bacteria. However, treatment with 200 and 400 μg mL^{-1} GA inhibited the development and growth of the pathogenic bacteria at concentrations of 10^6 and 10^7 CFU mL^{-1}, but these GA treatments could not completely suppress the growth of bacteria at 10^8 CFU mL^{-1}.

The cell wall maintains the inherent morphology of cells and exchanges substances. When the cell wall of a bacterium has been disrupted by an antimicrobial substance, the cell permeability is altered, materials can leak from the cell (Bush, 2012). Electron micrographs showed that GA-treated *Xoc* displayed damaged cell walls that showed signs of pits and irregular vesicles on the outer surface. A similar finding was reported by Helander et al, (2001). Furthermore, GA treatment resulted in a higher culture liquid electrical conductivity than that of the control. Thus, cell wall damage is at least one mechanism by which *Xoc* was negatively affected by GA.

An essential function of the cell membrane is serving as a selectively permeable barrier. The exposure to high concentrations of antimicrobial agents can alter the permeability of bacterial cell membranes and hinder normal bacterial metabolism (Bush, 2012). Our experimental results showed that GA enhanced the conductivity of the culture medium, which led to cytoplasm leakage and resulted in the leakage of intracellular DNA and RNA. These phenomena are similar to those reported for *Pseudomonas fluorescens* treated with GA (Lu et al, 2015), suggesting that GA could negatively affect *Xoc* cell wall integrity. Furthermore, the fluorescence intensity of cells treated with GA decreased to 58.10%, indicating that the cytosolic contents had leaked out from cells. In addition, the activity of intracellular LDH in the bacterial suspension treated with GA was higher than that of control, suggesting that LDH diffused outside the cell.

Our early works have demonstrated that GA could control rice bacterial leaf streak, with control efficacy of 64.54% in field(Wang et al, 2018). GA derivatives, methyl gallate, had ati-bacteria activity against *Ralstonia solanacearum*, *Pseudomonas syringae pv. lachrymans* and *Pectobacterium carotovora* subsp. *carotora*(Yuan et al, 2012) , and could effectively reduce the incidence of tomato bacterial wilt in the field(Li et al, 2014). Meawhile, four gallic acid derivatives have also ati-bacteria activity against *Ahernaria mali*, *Physalospora Piricola*, *Rhizoctonia solani* and *Phytophthora infestans*(Tan et al, 2018) . Therefore, GA and its derivatives have potential to be developed as a new pesticide to control plant disease.

In conclusion, the results suggest that GA altered *Xoc* membrane permeability and disrupts membrane integrity. Increasing concentrations of GA resulted in more evident damage. The results showed that the cell membrane was a target for bacterial growth inhibition.

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